Transbilayer Complementarity of Phospholipids in Cholesterol-Rich Membranes

Jianbing Zhang, Bingwen Jing, Nobuya Tokutake, and Steven L. Regen*

Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015

Received August 12, 2004; Revised Manuscript Received October 22, 2004

ABSTRACT: Lipid−lipid interactions across cholesterol-rich phospholipid bilayers were investigated by measuring nearest-neighbor preferences of exchangeable phospholipids derived from 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), in the presence of nonexchangeable dimers (i.e., templates) made from DMPE or DSPE. When homotemplates were present, a significant preference for homophospholipid association was observed. In contrast, when the corresponding heterotemplate was present, heterodimer formation was favored. These results support a model in which the longer phospholipid in one monolayer preferentially associates with the shorter one in the adjoining monolayer. In the absence of cholesterol, transbilayer complementarity was also observed but to a lesser degree. Transbilayer complementarity of phospholipids is likely to play an important role in stabilizing biological membranes and in promoting a compositional interdependence of their two lipid leaflets.

One of the most challenging, and perhaps one of the most important, issues that remains to be clarified in membrane biochemistry involves the possible existence of *transbilayer communication*; that is, a preference of one phospholipid to favor another phospholipid as a nearest neighbor in the adjoining monolayer. Much less challenging has been the determination of transbilayer asymmetry and lateral organization in natural and model membranes. Thus, it is now clear that there are significant differences in composition between the two halves of biological membranes (*1*, *2*). In eukaryotic cells, for example, phosphatidylcholines and sphingomyelins are favored in the outer leaflet, while phosphatidylserines and phosphatidylethanolamines are favored in the inner leaflet. How cholesterol is distributed between the two leaflets, however, has proven difficult to quantify (*3*−*5*). Differences in the degree of unsaturation and fluidity have also been detected between the inner and outer leaflets of biological membranes. In particular, the inner leaflet appers to be richer in unsaturated fatty acids and is more fluid (*6*−*10*). On the basis of a variety of physical and chemical measurements that have been made with natural membranes and model systems, there is a growing body of evidence indicating that cholesterol favors high-melting lipids as nearest neighbors, forming "condensed complexes" or "lipid rafts" (*11*−*28*). This lateral organization is thought to play an important role in fundamental cellular processes such as signal transduction and membrane trafficking.

How transbilayer asymmetry relates to lateral organization has remained a mystery in the physiologically relevant fluid phase. While some researchers have postulated the existence of transbilayer communication among the lipids, experimental support for this concept has been lacking (*17*). Recently, we provided the first evidence for transbilayer complementarity in a model membrane, using the nearest-neighbor recognition (NNR)[1] method (*29*−*31*). Specifically, we showed that liposomes composed of "long" and "short" phospholipids favor a transbilayer arrangement in which the short phospholipid lies directly across from the long one (Chart 1). In essence, the phospholipids complement each other by forming a smooth surface that maximizes hydrophobic interactions.

The primary aim of the work reported herein was to determine whether transbilayer complementarity also exists in *cholesterol-rich* phospholipid membranes. Since cholesterol is known to have a strong condensing effect on fluid membranes, it was not obvious whether such complementarity would be observed (*1*, *12*, *32*−*34*). Specifically, one can imagine that in a more condensed state, stronger hydrophobic interactions within each monolayer would render transbilayer interactions and surface roughness of lesser importance to the overall stability of the membrane. Given the large quantities of cholesterol that are found in mammalian membranes, we were compelled to address this biologically relevant question.

EXPERIMENTAL PROCEDURES

Phospholipid Dimers A′A′, AA, AB, BB, and B′B′. Each of these dimers was synthesized by methods similar to those previously described (*29*, *35*). The purity of each lipid was >99%, as determined by high-performance liquid chromatographic (HPLC) analysis.

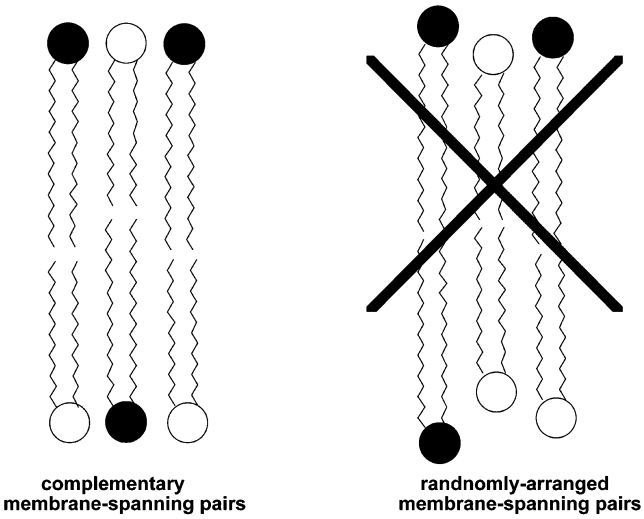
Heterodimer A′B′. A heterodimer template (A′B′) was synthesized according to the reaction scheme that is shown in Scheme 1. Thus, to a solution containing 41 mg (0.20 mmol) of 2,2′-(ethylenedithio)diacetic acid and 11.5 mg (0.1 mmol) of *N*-hydroxysuccinimide (NHS) in 15 mL of tet-

* Corresponding author: tel (610)758-4842; fax (610) 758-6560; email slr0@lehigh.edu.

[1] Abbreviations: DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; MALDI, matrix-assisted laser desorption ionization; NNR, nearest-neighbor recognition.

10.1021/bi048258f CCC: $30.25
Published on Web 02/12/2005

Chart 1



rahydrofuran (THF) was added 21 mg (0.1 mmol) of dicyclohexylcarbodiimide (DCC). After the mixture was stirred for 4 h at room temperature, the solvent was removed under reduced pressure and the residue was dissolved in 5 mL of chloroform. After the insoluble urea was removed by filtration, 63.5 mg (0.1 mmol) of DMPE and 41 μL (0.30 mmol) of triethylamine were added. After the product mixture was stirred for 5 h at room temperature, it was diluted with 20 mL of chloroform and washed with 30 mL of 10% HCl. The organic layer was separated and dried over anhydrous $MgSO_4$. The solvent was then removed under reduced pressure and the residue was purified by preparative thin-layer chromatography [silica gel, ($CHCl_3$/CH_3OH/H_2O, 65/24/4 v/v/v)] to give 54 mg (65%) of the half-reacted acid (A′X), having $R_f = 0.35$ and ^{1}H NMR (CD_3OD, 500 MHz) 0.84 (t, 6 H), 1.23 (m, 40 H), 1.56 (m, 4 H), 2.30 (hex, 4 H), 2.79−2.86 (hex, 4 H), 3.23 (s, 2 H), 3.30 (s, 2 H), 3.56 (s, 2 H), 4.14 (m, 5 H), 4.32 (m, 1 H), 5.22 (m, 1 H), 7.69 (s, 1 H), 9.92 (s, 1 H).

To a solution of 54 mg (0.065 mmol) of A′X and 9 mg (0.078 mmol) of NHS in 4 mL of chloroform was added 16 mg (0.078 mmol) of DCC. After the mixture was stirred for 2 h at room temperature, 50 mg (0.0668 mmol) of DSPE and 27 μL (0.196 mmol) of triethylamine were directly added. The mixture was stirred for an additional 4 h and then diluted with 20 mL of chloroform and washed with 30 mL of 10% HCl. The organic layer was separated and dried over anhydrous $MgSO_4$. The solvent was then removed under reduced pressure and the residue was purified by preparative thin-layer chromatography (silica gel, $CHCl_3$/CH_3OH/H_2O, 30/10/1 v/v/v) to give 75 mg (74%) of the desired heterodimer (A′B′) having $R_f = 0.44$ and ^{1}H NMR (CD_3OD, 500 MHz) 0.85 (t, 12 H), 1.23 (m, 96 H), 1.55 (m, 8 H), 2.28 (q, 8 H), 2.78 (s, 4 H), 3.26 (s, 4 H), 3.46 (s, 4 H), 3.88 (m, 8 H), 4.12 (m, 2 H), 4.38 (m, 2 H), 5.18 (s, 2 H), 7.98 (s, 2 H). Calcd for $C_{80}H_{152}N_2O_{18}P_2S_2Na_2$: MALDI [M − 2H + 3Na]$^+$ 1624. Found: 1624. The purity of A′B′ was >99% as determined by HPLC analysis.

Nearest-Neighbor Recognition Measurements. In a typical liposome preparation, a test tube was charged with a chloroform solution that contained 0.15 μmol of A′A′, 0.15 μmol of AA, 0.30 μmol of BB, and 0.49 μmol of cholesterol. The chloroform was then evaporated by passing a stream of argon over the solution. The lipid mixture was then dissolved in 100 μL of chloroform and diluted with 270 μL of diisopropyl ether. Subsequent addition of 33 μL of 3.3 mM Tris-HCl buffer [3.3 mM Tris-HCl, 50 mM NaCl, 0.67 mM NaN_3, and 0.33 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4] produced an emulsion. After the emulsion was sonicated for 3 min, in a mild (bath-type) sonicator, the organic phase was removed by gentle evaporation at 60 °C, resulting in a white gel at the bottom of the test tube. The gel was then collapsed by vigorous vortex mixing for 20−30 min, and 2.0 mL of additional 10 mM Tris-HCl buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM NaN_3, and 1 mM EDTA, pH 7.4) was added dropwise with vortex mixing. The dispersion was then degassed with an aspirator for 5 min, and the residual traces of organic solvent were removed by dialysis [Spectra/Por Membrane, molecular weight cutoff (MWCO) 6000−8000] under an argon atmosphere, against three 200 mL portions of degassed 10 mM Tris-HCl buffer, pH 7.4) over the course of 18 h. Large vesicles formed under these conditions were typically 1000 nm in diameter (dynamic light scattering).

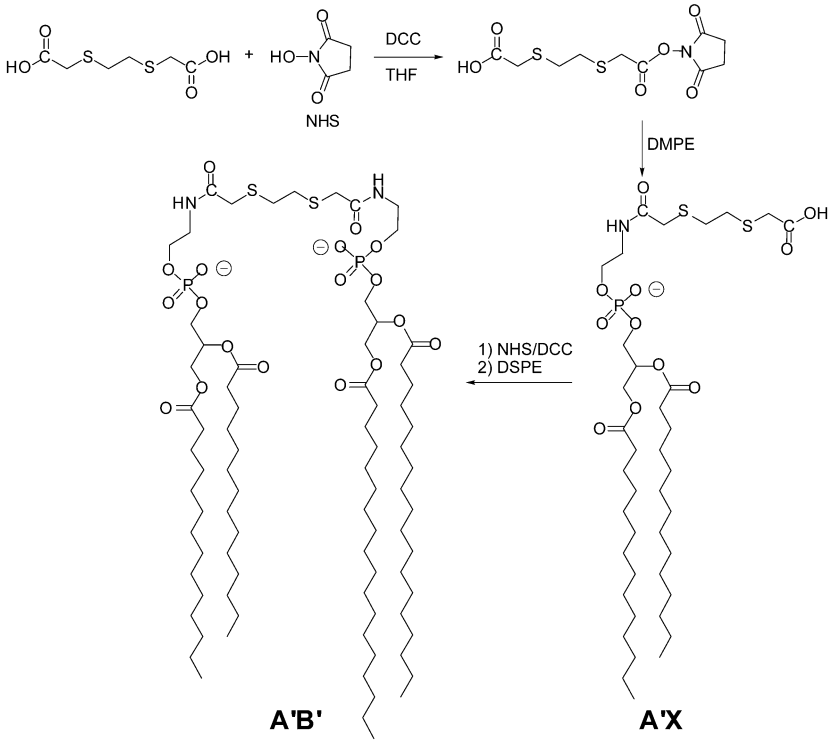
Thiolate−disulfide interchange reactions were initiated, after the dispersions were equilibrated at 60 °C, by injecting 25.5 μL of a 10 mM Tris buffer solution of 37.65 mM *threo*-dithiothreitol (0.96 μmol) and 24 μL of a Tris buffer solution that was 0.42 μM in monensin (10.2 pmol), with brief vortex mixing, and finally increasing the pH to 8.5 via addition of ca. 10 μL of 1.0 M NaOH. All dispersions were maintained under an argon atmosphere throughout the course of the interchange reactions. Aliquots (0.30 mL) were withdrawn as a function of time and quenched with 80 μL of 30 mM HCl (final pH 5.0). After removal of water under reduced pressure, the residue was dissolved in 2 mL of chloroform and centrifuged for 20 min. The chloroform layer was then removed under reduced pressure to yield a clear film, which was, subsequently, dissolved in 10 μL of chloroform and 90 μL of the mobile phase that was used for HPLC analysis.

Product mixtures were analyzed by C18 reverse-phase HPLC by use of a mobile phase that was composed of 10 mM tetrabutylammonium acetate in denatured ethanol/water/hexane (82/11/7) with a flow rate of 0.9 mL/min. The column was maintained at 31 °C and the components were monitored at 205 nm by a Waters 996 photodiode-array UV detector. Because A′A′ overlapped with AA in the HPLC traces, equilibrium constants were determined from peak area ratios of [AB]/[BB**]** and equations that appear in the Supporting Information section. Similarly, when B′B′ was present as a template, equilibrium constants were determined from peak area ratios of [AB]/[AA**]**. When the heterodimer template (A′B′) was employed, equilibrium constants were determined from peak area ratios ([AB + A′B′])/[BB**]** and equations that appear in the Supporting Information section. Analysis of product mixtures by quantitative thin-layer chromatography, with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] as a means of detection, indicated that the amount of exchangeable phospholipid present in the thiol monomer form was <0.6%.

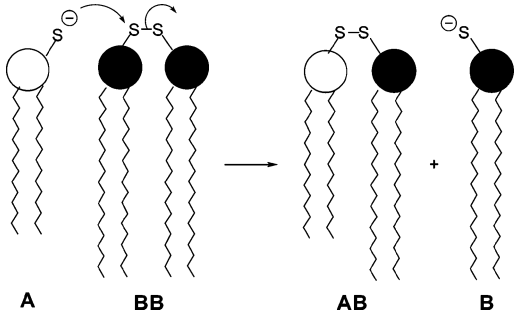
RESULTS AND DISCUSSION

Nearest-Neighbor Recognition Method. As discussed elsewhere, NNR measurements take molecular-level snap-

Scheme 1



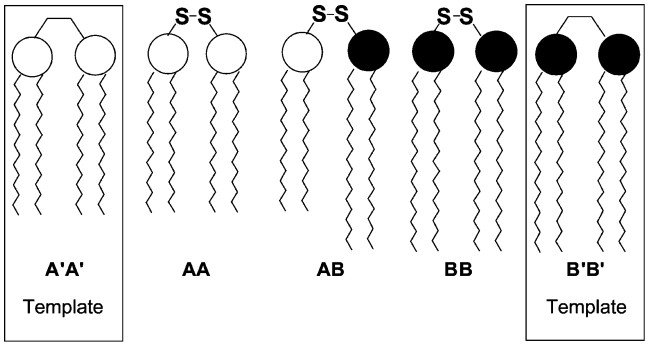
Scheme 2

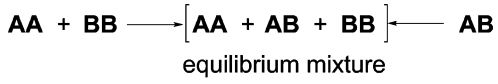


shots of membrane organization by detecting and quantifying the tendency of specific lipids to become nearest neighbors (*14*, *30*, *31*). Experimentally, equilibrium mixtures of exchangeable lipid dimers (i.e., dimers having monomer units that can undergo exchange with neighboring lipid monomers) are generated via thiolate−disulfide interchange reactions (Scheme 2). The interchange of monomers A and B among AA, BB, and AB is then governed by the equilibrium constant $K = [AB]^2/([AA][BB])$, which characterizes their mixing behavior (Chart 2). Thus, $K = 4.0$ when A and B mix ideally, $K < 4$ when homophospholipid associations are favored, and $K > 4$ when heterophospholipid associations are favored.

Probing Transbilayer Complementarity via Nearest-Neighbor Recognition. The variation of the NNR method that we have developed for probing transbilayer complementarity uses nonexchangeable dimers as templates (*29*). In essence, NNR experiments are carried out where either a part of A is substituted by a nonexchangeable dimer A′A′ or part of B by B′B′. If A and B are favored membrane-spanning pairs, then the inclusion of B′B′ in the membrane enhances the formation of AA in the adjoining monolayer leaflet by acting as a template (Chart 3). In this case, NNR

Chart 2





$$AA + BB \rightleftharpoons 2\,AB \quad (1)$$

$$K = [AB]^2/([AA][BB]) \quad (2)$$

experiments measure lateral interactions within each monolayer as well as transbilayer interactions, directly. In the same way, the introduction of A′A′ is expected to selectively stabilize BB in an adjoining monolayer. In principle, both A′A′ and B′B′ should generate a similar level of stabilization and, hence, similar $K < 4$ values.

Exchangeable and Nonexchangeable Phospholipid Dimers. In the present investigation, we used the exchangeable and nonexchangeable dimers that are shown in Chart 4. As noted previously, the gel to liquid-crystalline phase transition temperatures (T_m) for A′A′ and B′B′ are 21.1 and 55.6 °C, respectively, which are similar to those of AA ($T_m = 22.7$ °C) and BB ($T_m = 55.4$ °C); AB has a T_m of 33.9 °C (*35*). By use of procedures that are described under Experimental Procedures, large vesicles were prepared by reverse-phase

Chart 3

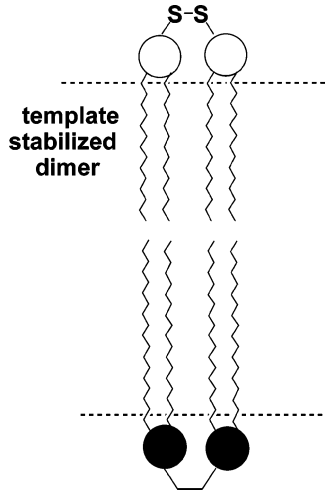
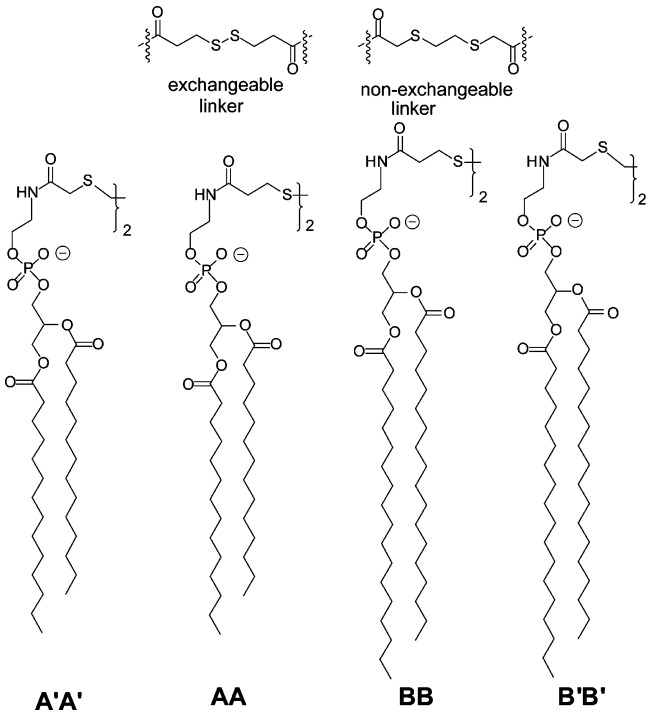


Chart 4



methods with the dimer and cholesterol compositions that are listed in Table 1. To preserve the thickness of the bilayer, A and B were always substituted by a mole-of-phosphorus-equivalent amount of the nonexchangeable analogues. Nearest-neighbor recognition measurements were then carried out by promoting thiolate−disulfide interchange reactions. To ensure that equilibrium had been reached, product mixtures were obtained from vesicles that were prepared from homodimers, as well as from heterodimers or a mixture of heterodimer plus homodimer.

Because A′A′ and AA overlapped in the HPLC traces, equilibrium constants were determined by measuring the molar ratio of AB/BB (see Supporting Information). Similarly, when B′B′ was used as the template, the molar ratios of AB/AA were used to calculate equilibrium constants. In Figure 1a, we show the dimer ratios that were observed as a function of time, with A′A′ as the template in the presence of 29 mol % cholesterol. Dimer ratios that were observed when B′B′ was used as the template in the presence of 29 mol % cholesterol are shown in Figure 1b.

Table 1: Template Effects on Dimer Equilibria

entry	template[a] type	template[a] mol %	AA[a] (mol %)	AB[a] (mol %)	BB[a] (mol %)	Ch[b] (mol %)	K[c]
1			50.0	00.0	50.0		3.99 ± 0.03[d]
			00.0	100	00.0		
2	A′A′	25.0	25.0	00.0	50.0		2.82 ± 0.15[d]
			00.0	50.0	25.0		
3	B′B′	25.0	50.0	00.0	25.0		2.76 ± 0.20[d]
			00.0	50.0	25.0		
4			50.0	00.0	50.0	29	2.27 ± 0.01
			00.0	100	00.0	29	
5	A′A′	25.0	25.0	00.0	50.0	29	1.22 ± 0.06
			00.0	50.0	25.0	29	
6	B′B′	25.0	50.0	00.0	25.0	29	0.89 ± 0.04
			00.0	50.0	25.0	29	
7	A′B′	25.0	37.5	00.0	37.5		5.97 ± 0.15
			00.0	75.0	00.0		
8	A′B′	25.0	37.5	00.0	37.5	29	4.50 ± 0.10
			00.0	75.0	00.0	29	

[a] Starting mol % of phospholipid dimers [b] Mol % of cholesterol included in the membrane, where each mole of phospholipid dimer is counted as two moles of lipid. Note: When cholesterol is included in the membrane, the total mol % of phospholipid (exchangeable + template) is equal to 71%, and cholesterol is equal to 29%. In this case, the mole percentages that are shown for the phospholipids apply, where the total equals 100%. [c] Equilibrium constants calculated from eq 2 (± 1 SD) are averages from both sets of experiments; equilibrium was reached in all cases within 3 h at 60 °C. [d] Reference 29.

Equilibrium constants that are reported in Table 1 were calculated from averages of the two sets of convergent data. In the absence of templates, A and B mix ideally, as reflected by a K value of 3.99 ± 0.03 (Table 1, entry 1) (*29*). In contrast, when 50% of AA was replaced by A′A′ (i.e., when 25 mol % A′A′ was used) K was lowered to 2.82 ± 0.15 (entry 2), and when 50% of BB was replaced by B′B′, K was found to be 2.76 ± 0.20 (entry 3) (*29*). Thus, the template effect by A′A′ and B′B′ in promoting homodimer formation corresponded to a $\Delta\Delta G°$ of 230 and 240 cal/mol, respectively. Analogous experiments that were carried out in the presence of 29 mol % cholesterol (in the absence of templates) led to a significant increase in the preference for homodimer formation (entry 4). As we have shown elsewhere, this effect is due to preferential association of the sterol with two or more of the longer (higher-melting) exchangeable phospholipids (i.e., B) (*14*, *15*). Similar experiments that were performed, where 50% of AA was replaced with A′A′ (i.e., when 25 mol % A′A′ was used), showed a *significant enhancement* in transbilayer complementarity. In this case, the value of K was reduced from 2.27 ± 0.01 to 1.22 ± 0.06 (entry 5), corresponding to a $\Delta\Delta G°$ of 410 cal/mol. When B′B′ was used as the template under the same conditions, K was reduced to a value of 0.89 ± 0.04 (entry 6), corresponding to a $\Delta\Delta G°$ of 620 cal/mol.

The stronger influence that both of these templates have in promoting homodimer formation in the presence of cholesterol is a likely consequence of preferred association of the sterol with the longer phospholipids B and B′B′. Specifically, such a preference is expected to favor their unraveling and elongating (i.e., cholesterol's condensing effect) relative to the shorter phospholipids, leading to an increase in chain-length mismatch and a greater driving force for transbilayer complementarity. The stronger effect by B′B′

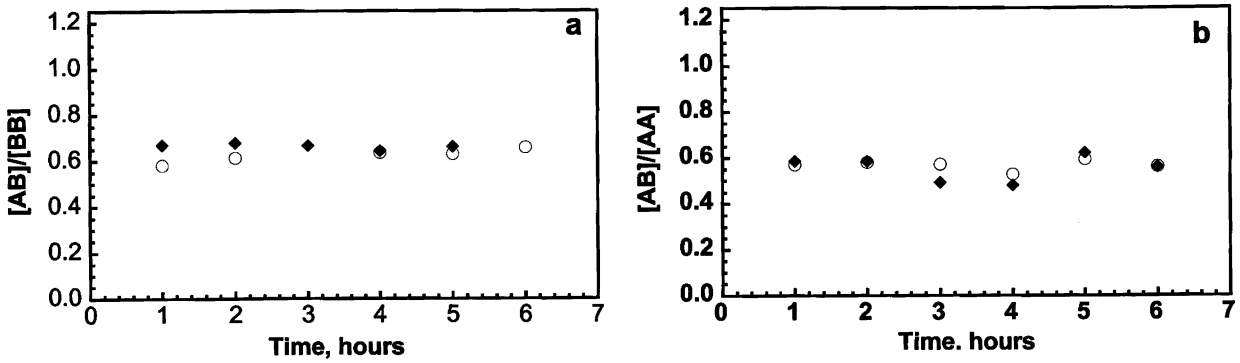


FIGURE 1: (a) Molar ratio of [AB]/[BB] as a function of time, starting from (○) homodimers and (◆) homodimers + heterodimers in cholesterol-rich bilayers containing A′A′. (b) Molar ratio of [AB]/[AA] as a function of time, starting from (○) homodimers and (◆) homodimers + heterodimers in cholesterol-rich bilayers containing B′B′.

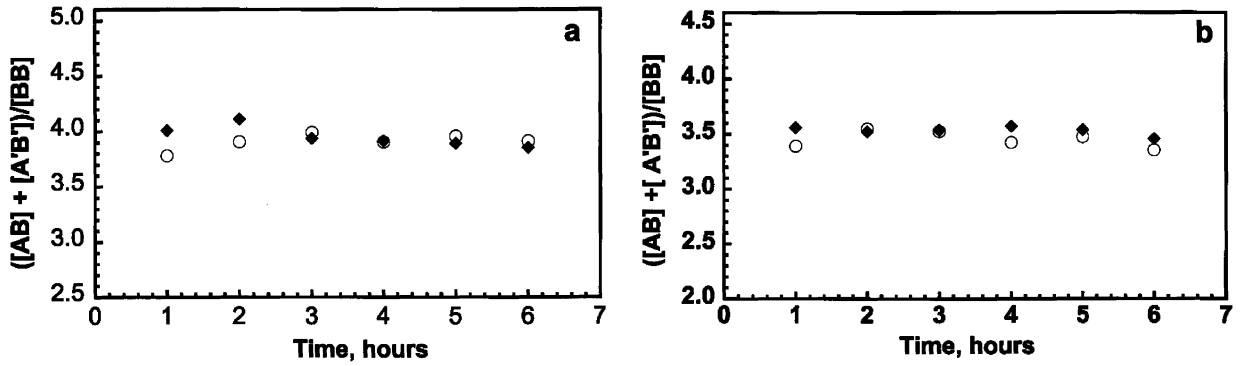


FIGURE 2: Molar ratio of ([AB] + [A′B′])/[BB] as a function of time, starting from (○) homodimers and (◆) heterodimers in bilayers containing A′B′ in which cholesterol is (a) absent and (b) present.

(i.e., the lower K value relative to that found with A′A′) is a likely consequence of an additional contribution from lateral interactions; that is, B′B′ stabilizes the condensed complexes formed between cholesterol and B more than A′A′ stabilizes those formed between A with cholesterol.

As further evidence for the validity of our method, we carried out experiments with a nonexchangeable *heterodimer* template (A′B′) in the absence and in the presence of cholesterol. In principle, this template should induce *a preference for heterodimer formation* among the exchangeable phospholipids. The synthesis of A′B′ is given under Experimental Procedures and will not be discussed here. Since A′B′ overlaps with AB in the HPLC chromatogram, we have used the molar ratio of ([AB] + [A′B′])/[BB] to determine the dimer equilibrium constants (see Supporting Information). In Figure 2 are shown these dimer ratios in the absence (a) and presence (b) of 29 mol % cholesterol. On the basis of these data, the value of K in the absence of cholesterol is 5.95 ± 0.15, which corresponds to a $\Delta\Delta G°$ of −270 cal/mol relative to that found in the absence of the template (i.e., entry 7 versus entry 1). Similarly, when this heterotemplate is included in bilayers that contain cholesterol, the value of K is raised to 4.50 ± 0.10, corresponding to a $\Delta\Delta G°$ of −450 cal/mol (i.e., entry 8 versus entry 4). Taken together, these results provide compelling evidence for the validity of our method.

Biological Significance. A large body of evidence currently exists that supports the presence of transbilayer asymmetry and lateral organization in eukaryotic cell membranes. Recent studies in model systems have confirmed that cholesterol favors high-melting lipids as nearest neighbors. The present findings are significant because they show, for the first time, that phospholipids in cholesterol-rich membranes complement one another across the bilayer, thereby minimizing surface roughness and maximizing hydrophobic interactions. Such "interleaflet communication" is likely to exist in natural membranes as well. Thus, one may now expect that structural or compositional modifications of one leaflet of a mammalian membrane will bring about a compensating modification in the adjoining leaflet. In a broader context, complementarity of phospholipids is likely to play a major role in stabilizing biological membranes.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health (PHS GM56149).

SUPPORTING INFORMATION AVAILABLE

Equations used to determine dimer equilibrium constants based on dimer ratios. This information is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Gennis, R. B. (1989) *Biomembranes: Molecular structure and function*, Springer-Verlag, New York.
2. Devaux, P. F., and Morris, R. (2004) Transmembrane asymmetry and lateral domains in biological membranes, *Traffic 5*, 241−246.
3. Schroeder, F., Nemecz, G., Wood, W. G., Joiner, C., Morrot, G., Aryault-Jarrier, M., and Devaux, P. F. (1991) Transmembrane distribution of sterol in the human erythrocyte, *Biochim. Biophys. Acta 1066*, 183−192.
4. Muller, P., and Hermann, A. (2002) Rapid transmembrane movement of spin-labeled steroids in human erythrocytes, *Biophys. J. 82*, 1418−1428.

5. Steck, T. L. YeJ, and Lange, Y. (2002) Probing red cell membrane cholesterol movement with cyclodextrin, *Biophys. J. 83*, 2118−2125.
6. Seigneuret, M., Zachowski, A., Hermann, A., and Devaux, P. F. (1984) Asymmetric lipid fluidity in human erythrocyte membrane: new spin-labeled evidence, *Biochemistry 23*, 4271−4275.
7. Marrot, G., Cribier, S., Devaux, P. F., Geldwerth, D., Davoust, J., Bureau, J. F., Fellmann, P., Herve, P., and Frilley, B. (1986) Asymmetric lateral mobility of phospholipids in the human erythrocyte membrane, *Proc. Natl. Acad. Sci. U.S.A. 83*, 6863−6867.
8. Cribier, S., Morrot, G., Neumann, J. M., and Devaux, P. F. (1990) Lateral diffusion of erythrocyte phospholipids in model membranes comparison between inner and outer leaflet components, *Eur. Biophys. J. 18*, 33−41.
9. EI Hage Chahine, M., Cribier, S., and Devaux, P. F. (1993) Phospholipids transmembrane domains and lateral diffusion in fibroblasts, *Proc. Natl. Acad. Sci. U.S.A. 90*, 447−451.
10. Julien, M., Tournier, J. F., and Tocanne, J. F. (1993) Differences in the transbilayer and lateral motions of fluorescent analogues of phosphatidylcholine and phosphatidylethanolamine in the apical plasma membrane of bovine aortic endothelial cells, *Exp. Cell Res. 208*, 387−397.
11. Radhakrishnan, A., and McConnell, H. M., (1999) Cholesterol−Phospholipid Complexes in Membranes, *J. Am. Chem. Soc. 121*, 486−487.
12. McConnell, H. M., and Radhakrishnan, A. (2003) Condensed complexes of cholesterol and phospholipids, *Biochim. Biophys. Acta 1610*, 159−173.
13. Veatch, S. L., and Keller, S. L. (2002) Organization in Lipid Membranes Containing Cholesterol, *Phys. Rev. Lett. 89*, 268101−268104.
14. Sugahara, M., Uragami, M., and Regen, S. L. (2003) Selective Association of Cholesterol with Long-Chain Phospholipids in Liquid-Ordered Bilayers: Support for the Existence of Lipid Rafts, *J. Am. Chem. Soc. 125*, 13040−13041.
15. Sugahara, M., Uragami, M., Yan, X., and Regen, S. L. (2001) The Structural Role of Cholesterol in Biological Membranes, *J. Am. Chem. Soc. 123*, 7939−7940.
16. Cao, H., Tokutake, N., and Regen, S. L. (2003) Unraveling the Mystery Surrounding Cholesterol's Condensing Effect, *J. Am. Chem. Soc. 125*, 16182−16183.
17. Simons, K., and Ikonen, E. (2000) How Cells Handle Cholesterol, *Science 290*, 1721−1726.
18. Ono, A., and Freed, E. O. (2001) Plasma membrane rafts play a critical role in HIV-1 assembly and release, *Proc. Natl. Acad. Sci. U.S.A. 98*, 13925−13930.
19. Dietrich, C., Volovyk, Z. N., Levi, M., Thompson, M. L., and Jacobson, K. (2001) From the Cover: Partitioning of Thy-1, GM1, and cross-linked phospholipid analogues into lipid rafts reconstituted in supported model membrane monolayers, *Proc. Natl. Acad. Sci. U.S.A. 98*, 10642−10647.
20. Edidin, M. (2003) The State of Lipid Rafts: From Model Membranes to Cells, *Annu. Rev. Biomol. Struct., 32*, 257−283.
21. Anderson, R. G., and Jacobson, K. (2002) A Role for Lipid Shells in Targeting Proteins to Caveolae, Rafts, and Other Lipid Domains, *Science 296*, 1821−1825.
22. Morris, R., Cox, H., and Mombelli, E., (2004) in *Membrane dynamics and domains* (Quinn, P. J., Ed.) pp 35−118, Kluwer Academic/Plenum Publishers, London.
23. Gaus, K., Gratton, E., Kable, E. P., Jones, A. S., Gelissen, I., Kritharides, L., and Jessup, W. (2003) Visualizing lipid structure and raft domains in living cells with two-photo microscopy, *Proc. Natl. Acad. Sci. U.S.A. 100*, 15554−15559.
24. Schutz, G. J., Kada, G., Pastushenko, V. P., and Dchindler, H. (2000) Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy, *EMBO J. 19*, 892−901.
25. Dietrich, C., Yang, B., Fujiwara, T., Kusumi, A., and Jacobson, K. (2002) Relationship of lipid rafts to transient confinement zones detected by single tracking, *Biophys. J. 82*, 274−284.
26. Subczynski, W. K., and Kusumi, A. (2003) Dynamics of raft molecules in the cell and artificial membranes. Approaches by pulse EPR spin labeling and single molecule optical microscopy, *Biochim. Biophys. Acta 1610*, 231−243.
27. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction, *Nat. Rev. Mol. Cell Biol. 1*, 31−39.
28. Kunzelman-Marche, C., Freyssinet, J. M., and Martinez, M. C. (2002) Loss of plasma membrane phospholipid asymmetry requires raft integrity, *J. Biol. Chem. 277*, 19876−19881.
29. Zhang, J,. Jing, B., Tokutake, N., and Regen, S. L. (2004) Transbilayer Complementarity of Phospholipids. A Look Beyond the Fluid Mosaic Model, *J. Am. Chem. Soc*., *126*, 10856−10857.
30. Davidson, S. K. M. and Regen, S. L. (1997) Nearest-Neighbor Recognition in Phospholipid Membranes, *Chem. Rev. 97*, 1269−1280.
31. Regen, S. L. (2002) Lipid−lipid recognition in fluid bilayers: solving the cholesterol mystery, *Curr. Opin. Chem. Biol. 6*, 729−735.
32. Leathes, J. B. (1925) Role of fats in vital phenomena, *Lancet 208*, 853−856.
33. Ipsen, J. H., Karlstrom, G., Mouritsen, O. G., Wennerstrom, H., and Zuckermann, M. J. (1987) Phase equilibria in the phosphatidylcholine−cholesterol system, *Biochim. Biophys. Acta 905*, 162−172.
34. Rubenstein, J. L. R., Smith, B. A., and McConnell, H. M. (1979) Lateral Diffusion in Binary Mixtures of Cholesterol and Phosphatidylcholines, *Proc. Natl. Acad. Sci. U.S.A. 76*, 15−18.
35. Krisovitch, S. M., and Regen, S. L. (1992) Nearest-neighbor recognition in phospholipid membranes: a molecular-level approach to the study of membrane suprastructure, *J. Am. Chem. Soc. 114*, 9828−9835.

BI048258F